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Dietary Berries and Ellagic Acid Diminish Estrogen-Mediated Mammary Tumorigenesis in ACI rats

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Estrogen acts as a complete mammary carcinogen in ACI rats. Prevention studies in this model allowed us to identify agents that are effective against estrogen-induced mammary carcinogenesis. In this study, we investigated efficacy of dietary berries and ellagic acid to reduce estrogen-mediated mammary tumorigenesis. Female ACI rats (8–9 wk) were fed either AIN-93M diet ($n = 25$) or diet supplemented with either powdered blueberry ($n = 19$) and black raspberry ($n = 19$) at 2.5% wt/wt each or ellagic acid ($n = 22$) at 400 ppm. Animals received implants of 17ß-estradiol 2 wk later, were palpated periodically for mammary tumors, and were euthanized after 24 wk. No differences were found in tumor incidence at 24 wk; however, tumor volume and multiplicity were reduced significantly after intervention. Compared with the control group (average tumor volume = 685 ± 240 mm3 and tumor multiplicity = 8.0 ± 1.3), ellagic acid reduced the tumor volume by 75% ($P < 0.005$) and tumor multiplicity by 44% ($P < 0.05$). Black raspberry followed closely, with tumor volume diminished by > 69% ($P < 0.005$) and tumor multiplicity by 37% ($P = 0.07$). Blueberry showed a reduction (40%) only in tumor volume. This is the first report showing the significant efficacy of both ellagic acid and berries in the prevention of solely estrogen-induced mammary tumors.

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among women in the United States. Among the women diagnosed, over 53,000 women are expected to have ductal carcinoma in situ (DCIS) (1). DCIS remains one of the most commonly diagnosed breast cancers with up to 25% recurrence as invasive carcinomas (2). Prolonged exposure to physiological levels of 17ß-estradiol is considered as a key risk factor for the development of sporadic breast cancer (3,4). Furthermore, associations between the use of hormone-replacement therapy and development of breast cancer in postmenopausal women (1,4) delineates a role for estrogens in human breast cancer.

The use of carcinogen-induced mammary tumors in rats as a preclinical model for prevention studies is well documented. There are several animal models available with considerable heterogeneity in mammary tumors depending on the type of rat strain, carcinogen used, time and mode of carcinogen administration, and so forth (5–9). The use of any particular model is dependent on the hypothesis tested. Several key points support the use of the estrogen-induced mammary tumor model to study breast cancer prevention. Foremost, estrogen is clearly and undisputedly associated with the etiology of the disease in humans. Then, estrogen-induced tumors in ACI rats exhibit chromosomal instabilities, which are also often seen in human breast cancer (10–12). Further, the development of estradiol-induced and 7,12, dimethylbenze[*a*]anthtacene (DMBA)-induced carcinogenesis involve genetically distinct mechanisms (13). Although these rats are susceptible to estrogen-induced prolactinomas, the loci that control the pituitary and mammary tumor susceptibilities are genetically distinct (13–15). Also, reduction of circulating prolactin levels in these rats by administering a lower dose of estradiol increases both tumor volume and multiplicity (16). In addition, the chromosomes that are affected in estrogen-induced carcinogenesis are homologous to those that are affected in humans. Finally, tumors display molecular markers such as an overexpression of cyclin D1 and c-myc, similar to breast cancer pathology in humans (17). Thus, this model offers an apt in vivo system for testing preventive intervention strategies.

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Evidence from several observational studies have indicated a link between consumption of fruits and vegetable and reduced risk of cancer (18). Further, research on chemopreventive efficacy of agents such as ß-carotene in the prevention of preclinical cancer set the precedence for its application in clinical trials (19). The 2 major clinical trials α-tocopherol ß-carotene prevention trial (ATBC) in Finland and the ß-carotene and retinol efficacy trial (CARET) in the United States were done in smokers with an intervention dose of up to 30 mg ß-carotene and 25,000 IU of vitamin E compared to a recommended intake of 1.8 mg and 22 IU, respectively, for nonsmokers (20,21). Unexpectedly, the incidence of lung cancer in the high-dose intervention groups was higher than the placebo (20,22). Several issues that may have lead to the discrepancy between the observational and clinical studies, including the fact that these trials mostly concentrated on the effect of a single agent, whereas most epidemiological study correlations are the result of interactions between several whole food constituents (23,24). Among the various whole food sources available, berries are potential chemopreventive agents due to several reasons. First, berries are a good source of several chemopreventive nutrients including ß-carotene; selenium; vitamins A, C, and E; as well as phytochemicals such as lutien; ellagic acid; and anthocyanins (25–28). Second, several berries including those used in this study are native to the United States and are widely cultivated and commercially available. Third, berries are an integral part of the Western diet and can be easily incorporated into the existing dietary patterns of the public. Finally, berries are rich sources of phytochemicals that show chemopreventive efficacy as discussed below.

Ellagic acid is a polyphenol formed by the dimerization of gallic acid in various plants (29). It has been shown that ellagic acid may elicit cancer prevention by several mechanisms, which include direct binding to DNA, attenuation of carcinogen metabolism via the P450 pathway, and downregulation of cell-cycle activators and upregulation of proapoptotic mechanisms (30,31). Ellagic acid in plants is present as ellagitannins (32). It is released in the gut by the microflora and then absorbed as ellagic acid. Analysis of ellagic acid contents of various berries shows that although some commonly available berries, such as blackberry and raspberry, are rich sources (1,500 ppm each), others like blueberry (<100 ppm) are not (25). Several studies by Stoner and colleagues (33–35) have shown the protective effects of black raspberry on gastrointestinal tumors induced by chemical carcinogens. Although black raspberry and blueberry are good sources of anthocyanins, they differ significantly in their anthocyanin profile (28). Black raspberry is a rich source of cyanidin-, whereas blueberry contains 5 different anthocyanins and is especially rich in delphinidin (28). Blueberry has been much touted for its antioxidant properties, both historically and experimentally, due to its anthocyanin content (36,37). Also, experimental evidence suggests that different phytochemicals in a whole food source may potentiate each other's anticarcinogenic effects (38).

These facts formed the basis for the following rationale of our study. First, we examined the efficacy of relatively low doses of natural chemopreventive agents, such as ellagic acid and berries, in reducing estrogen-mediated mammary tumors in ACI rats so that maximum clinical relevance could be established from the results. Second, we provided both berries, a natural source of ellagic acid, and pure ellagic acid in the diet to distinguish the effects of "whole food" versus "active ingredient" in its biological response. Finally, we chose 2 berries that differed widely in their ellagic acid contents but had similar anthocyanin levels to evaluate the role of each of the antioxidant components in yielding benefits.

MATERIALS AND METHOD

Diets

All diets were ordered from Harlan-Teklad, Inc. (Madison, WI). The AIN-93M diet was supplemented with powdered berries (2.5% wt/wt) or ellagic acid (400 ppm). The concentration of berries used was the lowest effective dose against carcinogen-induced colon carcinogenesis based on published reports (33). The dose of ellagic acid was selected based on previous reports of the lowest dose effective in reducing N-nitrosomethyl benzylamine (NMBA)-induced esophageal tumors (39) and short-term studies in our laboratory (unpublished data). The equivalent content of ellagic acid in the blueberry and black raspberry diets, when supplemented at 2.5% (wt/wt), is approximately < 2.5 and 50 ppm, respectively (based on values of ellagic acid in parts per million of dry weight as published) (25,33).

Black raspberry was procured as a freeze-dried powder from Van Drunen farms (Momence, IL) through Dr. Gary Stoner of The Ohio State University (Columbus, OH). The processing of black raspberry was done as described (33). Organic blueberry was purchased from a local farm (Liberty, KY). Three different high bush cultivars of blueberry (*V. corymbosum L*)—Bluecrop, Berkeley, and Bluejay—were harvested in the morning, stored overnight below 10°C, and transported the next morning to the laboratory for processing. Berries were rinsed with distilled water and dehydrated using commercial food dehydrators (at 40°–60°C). The dried berries were finely powdered using a kitchen blender, sieved, and lyophilized to remove residual moisture. All berries were then vacuum packed and stored at –20°C until use. The 3 different cultivars of blueberry were mixed in equal ratios prior to mixing in diet to eliminate the need to test each cultivar individually and also to equalize the varying levels of phytochemicals present in each cultivar. Ellagic acid (>96% purity) was purchased from LKT labs (St. Paul, MN). The cornstarch and fiber components of the AIN-93M diet were replaced for the berry diets, based on the nutritional information available for each berry (http://www.nal.usda.gov/fnic/foodcomp), and a proximate analysis was performed to ascertain that the diets were isocaloric. The daily feed intake by animals was assessed

TABLE 1
Experimental Protocol[a]

Diet	17(ß-Estradiol)	No. Animals
Control diet-AIN 93M	−	6
	+	25
2.5% Blueberry	−	6
	+	19
2.5% Black raspberry	−	6
	+	19
400 ppm ellagic acid	−	6
	+	22

[a] In the study, 7- to 8-wk-old animals were received and maintained initially on AIN-93M control diet for a week followed by experimental diets for another 2 wk before 17ß-estradiol implantation. Animals were maintained on experimental diets until the end of the study.

by subtracting the unused diet from the initial amount provided per cage divided by the number of rats in the cage.

Animal Treatment and Assessment of Tumor Indices

Female ACI rats, 7 to 8 weeks old, were purchased from Harlan-Sprague-Dawley, Inc. (Indianapolis, IN), housed under ambient conditions, and had access to food and water ad libitum. Animals were acclimated for 1 wk on AIN-93M diet prior to randomizing them into different groups (Table 1). After feeding experimental diets for 2 wk, animals then received either a 3 cm silastic implant containing 27 mg 17ß-estradiol as described (16,40) or sham implants. Animals were weighed biweekly after estrogen implantation to track weight changes and disease progression. Starting at 12 wk after estrogen implantation, animals were palpated weekly for tumor appearance. The frequency of palpation was increased to twice a week, on appearance of the first tumor, to record tumor latency and incidence. The experiment was terminated after 24 wk of estrogen treatment. At termination, animals were euthanized and each animal was examined grossly for the presence of mammary tumors. Most mammary tumors were spheroids and could be clearly measured in 3 dimensions. Each tumor was measured in all 3 dimensions, after excision from the mammary pad, using calipers, and the tumor volume was calculated using the standard formula for the volume of a spheroid—$2/3\pi$ r1*r2*r3, where r1, r2, and r3 represent the radii of the tumor. The tumor volume per animal is the sum of the volumes of all individual tumors. All 6 mammary glands were harvested after the removal of the tumors and weighed together (Table 2). Care was taken to remove all glands without the inclusion of muscle or skin. Representative tumors from each animal were analyzed for histopathology to confirm that they were mammary adenocarcinomas.

Analysis of 17β-Estradiol Levels

Trunk blood was collected from animals after euthanasia, and the serum estradiol levels were measured by Roche E170 immunoassay analyzer using electrochemiluminescent detection.

Statistical Analysis

Experimental data were analyzed using the Statistical Analysis Software, SAS version 8. The longitudinal analysis of the data on body weights was carried out using the PROC MIXED procedure. A linear trend for weight change was established at P value < 0.0005. The differences in weight gains or losses between different groups were assessed using the same procedure, and for this analysis, a P value < 0.0001 was considered significant due to large number of weight comparisons at 13 biweekly time points. The tumor volume and multiplicity were compared using the General Linear Models (SAS procedure PROC GLM) and the Poisson Regression Model (SAS procedure PROC GENMOD) procedures, respectively, and a P value <0.05 was considered significant. The difference in the

TABLE 2
Effect of diet supplemented with black raspberries, blueberries, and ellagic acid on organ weights and tumor indexes in ACI rats treated with 17ß-estradiol[a]

Group	Animal Weight (9)	Mammary (g)	Liver (g)	Pituitary (g)	Tumor Volume $(mm)^3$	Tumor Multiplicity	Volume/ tumor $(mm)^3$
Control diet ($n = 11$)	169 ± 6.4	5.0 ± 0.3	3.84 ± 0.3	0.22 ± 0.03	685 ± 240	7.9 ± 1.3	115 ±39
Blueberry diet ($n = 16$)	159 ±5.6	4.7 ± 0.2	3.89 ± 0.3	0.19 ±0.01	409 ± 73	8.2 ± 1.0	45 ± 7
					$p < 0.835$	$p < 0.749$	$p < 0.170$
Black raspberry diet ($n = 11$)	162 ± 8.5	4.7 ± 0.3	4.41 ± 0.6	0.25 ± 0.02	211 ± 69	4.7 ± 0.7	38 ± 70
					$p < 0.003$	$p < 0.070$	$p < 0.034$
Ellagic acid diet ($n = 19$)	167 ± 4.3	4.9 ± 0.2	4.53 ± 0.3	0.19 ± 0.01	168 ± 34	4.5 ± 0.5	34 ±7
					$p < 0.001$	$p < 0.027$	$p < 0.009$

[a] Animals were euthanized after 24 wk of estrogen treatment. Organ wet weights were measured after excision. Tumor volume was calculated as the volume of a spheroid (2/3 π r1*r2*r3). Values denote mean ± standard error of measurement. "n" designates only those animals that survived 24 wk. All comparisons are between control diet and respective diets.

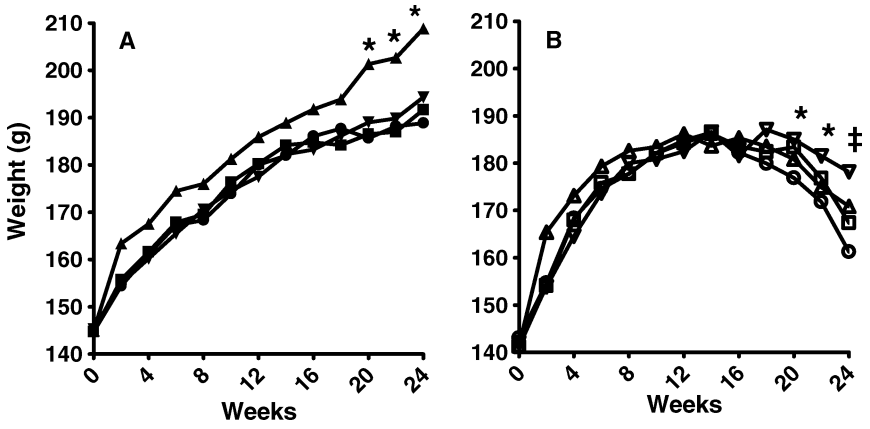

FIG. 1. Comparison of weight gain after E2 or SH implants. Animals were weighed every fortnight until termination of study. 1A -SH treated (closed symbols); 1B - E2 treated (open symbols). Control diet ●, ○; Blueberry diet ■, □; Black raspberry diet ▲, △; Ellagic acid diet ▼, ▽. Statistically significant weight differences are indicated. * - Statistically different from animals fed control diet ($p < 0.05$). ‡- Statistically different from animals fed control diet ($p < 0.005$).

mortality index was assessed using the nonparametric survival analysis techniques and the log-rank test.

RESULTS

Serum Estrogen Level

Serum 17β-estradiol levels for these animals were measured at 6 wk and 24 wk. At 6 weeks, the mean serum estrogen levels were significantly ($P < 0.0001$) elevated (194 $\pm$ 20 pg/ml) in the estradiol-treated versus sham group (35 $\pm$ 9 pg/ml). The levels further increased somewhat after 24 wk of treatment (236 $\pm$ 24 pg/ml), but the increase was insignificant compared to 6 wk. No significant change was found in age-matched controls (44 $\pm$ 7 pg/ml). There was no effect of dietary supplementation on serum estradiol levels at both 6 and 25 wk.

Effect of Estrogen Treatment and Experimental Diets on Body Weight

Measurement of diet intake showed no significant difference between various groups, suggesting that supplementation had no effect on diet intake. Furthermore, animals gained weight progressively irrespective of the implants; however, estrogen-treated animals gained more weight than their sham counterparts starting at 4 wk after the treatment, irrespective of the diet, indicating that this weight gain was a direct result of the estrogen treatment (Fig. 1B). Sham-treated animals on control diet continued to gain weight until the end of the study. Sham-treated animals receiving experimental diets also showed similar trends in weight gain, except that diet supplemented with black raspberry showed higher weight gain, starting as early as 2 wk after the experimental diet; but the difference was significant only after 20 wk of the dietary regimen ($P < 0.05$; Fig. 1A). Further, proximate analysis showed that all diets were isocaloric (data not shown).

Effect of Berry- and Ellagic Acid-Supplemented Diets on the Disease-Associated Weight Loss

At 22 and 24 wk, the difference in weight between estradiol- versus sham-treated animals on the same diet was significantly lower ($P < 0.0001$) for all groups. In contrast, none of the sham-treated groups lost weight until the end of the study suggesting that the weight loss was a disease-associated phenomenon in the estradiol-treated animals. Comparison of estradiol-treated groups on various diets revealed that animals fed a control diet lost the most weight, followed by animals fed blueberry-, black raspberry- and ellagic acid-supplemented diets (Fig. 1B). Ellagic acid-fed animals showed significant resistance to weight loss even toward the end of the study, that is, from the 20th ($P < 0.05$) to the 24th wk ($P < 0.005$). Thus, there was an intervention-associated prevention of weight-loss in all estradiol-treated animals, with ellagic acid-supplemented diet showing the most pronounced effect.

Effect of Estrogen Treatment and Experimental Diets on the Rate of Mortality

The morbidity in estradiol-treated groups was defined by weight loss of >7 g a week. In addition, other parameters such as loss of mobility, balance, grooming, the presence of eye deposits, and a dull hair coat were taken into account and scored subjectively on a scale of 1 to 5 (1 being the best and 5 being the worst). Animals that did worse (score > 3) on 3 or more of these criteria and also had rapid weight loss were euthanized. It was observed from previous studies in our laboratory that rats showing severe morbidity will eventually die in their cages and hence were preemptively euthanized in this study, and this was taken as indicator of the mortality (Fig. 2) Additionally, animals whose tumor size was between 1 and 1.5 cm in diameter were also euthanized. These animals, however, were excluded from the mortality index because they did not meet the morbidity criteria. Estrogen-treated animals on a control diet showed

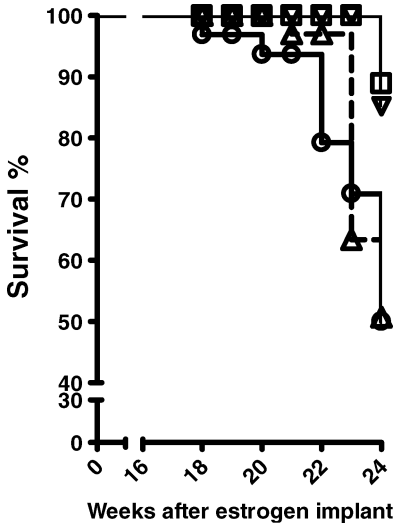

FIG. 2. Kaplan-Meier survival curves for ACI rats with estradiol implants fed different diets. Control diet, Blueberry diet, Black raspberry diet, Ellagic acid diet. All remaining animals were euthanized at 25 weeks.

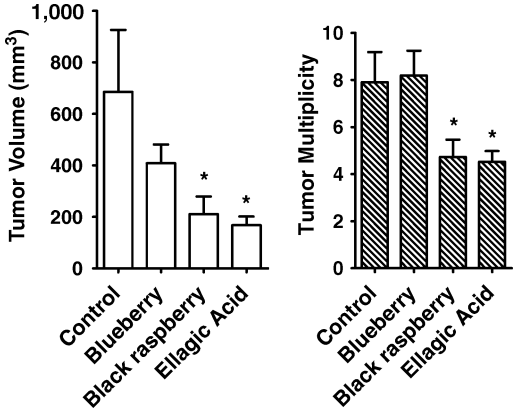

FIG. 3. Effect of experimental diets on tumor indices. The tumor multiplicity was compared with the GLM procedure and tumor volume was compared using the GENMOD procedure as described in methods. A p-value ≤ 0.05 was considered significant and is denoted by an asterisk.

the highest morbidity and mortality rate starting at 18 wk after the treatment. The survival rate in this group progressively declined, reaching < 50% after 24 wk, thus only 11 of 25 animals survived at the termination of the study. In contrast, all intervention groups were significantly different from the control diet (log rank test, P value < 0.005): Both ellagic acid- and blueberry-fed animals showed no morbidity and had > 85% survival at 24 wk. The group on black raspberry-supplemented diet initially showed a higher survival rate, but it declined rapidly and had 60% mortality at 24 wk. Although the ellagic acid group showed no sign of morbidity, 3 out of 22 animals had to be euthanized before 24 wk because of the large tumors. These data suggest that the disease progression, as measured by the incidence of morbidity, was significantly delayed by the intervention—by about 3 (black raspberry diet) to 6 (blueberry and ellagic acid diets) wk compared with the control group (Fig. 2).

Effect of Experimental Diets on Tumor Indices

The first palpable tumor was detected at 90 days after estradiol treatment without any intervention, with a mean tumor latency of 134 ± 6 days. The tumor development was marginally delayed in the intervention groups by 18, 20, and 21 days for animals fed blueberry-, black raspberry- and ellagic acid-supplemented diets, respectively. However, no difference in tumor latency, as measured by periodical palpation, was seen thereafter. Tumor incidence was 100% at 24 wk in all 17ß-estradiol-treated animals regardless of supplementation. On termination after 24 wk, the tumor multiplicity in the control diet group was 7.9 ± 1.3, and the tumor volume was 685 ± 240 (Table 2). All tumors were confirmed to be mammary tumors through histopathology (data not shown). The total tumor volume divided by the number of tumors was considered as the tumor burden per animal. Blueberry diet resulted in a 60% reduction in tumor burden without any change in tumor multiplicity. Black raspberry diet resulted in > 60% reduction ($P < 0.05$) in tumor burden and nearly 40% reduction in tumor multiplicity. Ellagic acid showed the highest reduction in tumor burden (> 70%; $P < 0.05$) and tumor multiplicity (> 43%; $P < 0.05$; Table 2, Fig. 3).

DISCUSSION

The failure of preventive trials with individual micronutrients has steered the scientific community toward appreciating the interaction between bioactive food components present in whole foods (24,41). Ellagic acid, present abundantly in many berries, is a known chemopreventive agent. It has been shown to successfully reduce the incidence and progression of carcinogen-induced tumors in the skin, lung, esophagus, liver, and colon, in rodents, when given orally (31). Several mechanisms such as antioxidant effect, modulation of detoxification enzymes, regulation of cell cycle pathways, DNA binding, and DNA repair pathways have been attributed to this (30,31).

However, in addition to ellagic acid, berries contain several other phytochemicals, including anthocyanins (28,35). Further, blueberry and black raspberry show high antioxidant activity (42,43). Black raspberry is known to affect inflammatory cellular pathways such as cyclooxygenase-2, nuclear factor-κB involved in tumor progression (44,45). Both ellagic acid and berry extracts inhibit in vitro proliferation of malignant cells through proapoptotic mechanisms (38,46,47). These findings suggest that berries have the potential to act via several anticarcinogenic pathways.

Although ellagic acid is moderately effective at widely ranging doses (1,000 ppm (48) and 8,000 ppm (49)) in preventing mammary tumor incidence induced by different chemical carcinogens (48,49), neither berries nor ellagic acid have been tested against estrogen-induced mammary carcinogenesis.

Evidence that both ellagic acid and several anthocyanins may act as a selective estrogen receptor mediators (50,51) warrant the use of these agents in mammary cancer chemoprevention and may partially explain their effect in the current study.

Among the different berries, black raspberry has the highest ellagic acid content, and blueberry has the lowest (25,33). Ellagic acid is released from the ellagitannins by the action of the gut microflora (32). There is both in vitro and in vivo evidence suggesting that a natural source of a pure compound is more effective in eliciting biological effects than the compound itself (38,52). The results from this study also support this observation. The level of ellagic acid in the black raspberry diet in this study is about 50 ppm (based on a 2.5% dietary dose with 2,000 ppm ellagic acid in 1 g of dry black raspberry), but it is highly effective in reducing tumor indices (Table 2). Pure ellagic acid, at about 8 times this dose, elicits the same response. Thus, either ellagic acid is more bioavailable from ellagitannins in black raspberry, or other components of black raspberry, such as anthocyanins as well as other flavanoids, work synergistically to offer better protection. There is support for the latter because blueberry, a poor source of ellagic acid but rich in anthocyanins, also elicits a moderate reduction in the estrogen mammary carcinogenicity as well a significant reduction in morbidity.

Disease progression can be understood as the decline in health of the animals as indicated by weight loss and increased morbidity score. We believe that the estradiol doses given to these animals may have been too high, and the subsequent toxicity induced by the estradiol levels may have a confounding effect on the actual effectiveness of these diets. Li and coworkers (53), in their initial work, reported that animals implanted with 3 mg cholesterol pellet had a serum estradiol levels < 145 pg/ml at 6 mo. These animals did not show high mortality albeit a marginal weight loss (53). In a recent report using the same model, other investigators (54) reported that animals suffered significant weight loss even at 20 wk. The serum estradiol levels in these animals were > 300 pg/ml at 6 and 12 wk. This suggests that a high serum estradiol level plays a significant role in inducing morbidity in the animals. It is clear from our results that although dietary interventions were highly effective in reducing weight loss and morbidity (Fig. 2), the doses of dietary intervention may have been insufficient to combat the effects of high circulating estradiol levels. Also, the varying effects of the 2 berries in preventing the morbidity may be related to the differences in their anthocyanin content as well as their anthocyanin profiles (Fig. 2) (28). Using an improved model, this laboratory has found that reduced serum levels of estradiol delivered by shorter estradiol silastic implants, which resulted in serum estradiol level of 200 ± 44 pg/ml, can produce 100% tumor incidence at the expense of somewhat longer duration (7–8 mo) (16). It remains to be determined if the berry and ellagic acid interventions will be more effective or effective even at lower doses than used in the present study when the estradiol dose is reduced.

In conclusion, this is the first study demonstrating significantly diminished estrogen-mammary carcinogenicity by dietary berries and ellagic acid. This also reveals the in vivo efficacy of berries in reducing tumorigenesis in an organ site other than the gut. In addition, due to the reasons discussed in the introduction, these results have high clinical relevance, especially in the prevention of breast cancer.

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